TOXIN-MEDIATED TRANSFER AND EXPRESSION OF GENES IN NERVE CELLS

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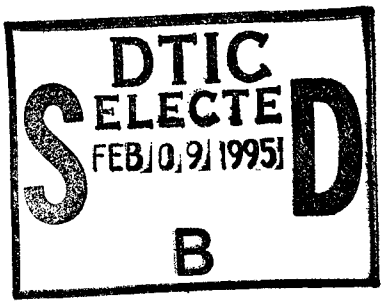

Final Report

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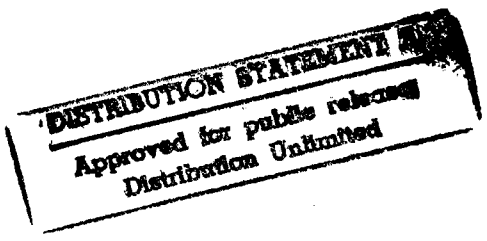

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13. ABSTRACT (Maximum 200 words)

Receptor-Mediated Gene Transfer In the CNS: A Feasibility Study This research sought to determine the feasibility of using receptor-mediated gene transfer as a mechanism for introducing the expression of foreign genes in nerve cells. DNA carrier systems were constructed using neuronal ligands that are rapidly internalized by receptor-mediated endocytosis. These proteins, principally **wheat germ agglutinin** and **tetanus toxin C-fragment**, were complexed with high expression reporter genes and applied to nerve cells in vitro, and administered in vivo into rats. Uptake and expression of the reporter genes were analyzed by standard enzymatic and histochemical procedures.

While we have demonstrated, for the first time, that cells in brain can internalize and express plasmid DNA, there is no evidence that this process can be made specific through the introduction of a receptor-mediated mechanism. The findings indicate that; (1) receptor-mediated uptake and expression does occur in the CNS, and (2) lysosomal degradation is probably not the underlying basis for our inability to observe expression. From this it may be concluded that receptor-mediated uptake is not an efficient means for directing the expression of foreign genes in nerve cells *in vivo*.

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4.A. STATEMENT OF THE PROBLEM STUDIED

4.A.1. Targeted Gene Delivery: Justification for the Project The introduction of foreign genes into cells is a powerful approach for controlling cell function. It is the underlying basis for viral infection and the rationale for scientific research on gene transfer and therapy. If gene therapy is to become a clinical reality, efficient and safe methods must be developed for precisely targeting the delivery of foreign genes into specific cells. An attractive potential for this approach is that the replacement of a single defective gene product will correct the complex spectrum of downstream consequences that underlie the pathophysiology of an inherited disorder.

Targeted gene delivery may also provide a means for arresting age-related processes that result from the under expression of trophic substances. Our current understanding predicts that enhanced production of neurotropins within the central nervous system will delay the progressive loss of neurons that normally occurs with aging. As such, introducing genes into nerve cells could enable us restore deficits that occur as a consequence of aging, use of neuroactive drugs, injury, or disease, as well as those of inherited disorders.[1-3]

This research investigated the process of receptor mediated endocytosis as a means to target the delivery of foreign genes into nerve cells. Regulation of cell function through receptor-targeted gene expression offers a degree of specificity in treatment that is not achieved by other approaches. First, receptor-mediated gene transfer targets the expression of foreign genes into only those cells that possess receptors for the carrier ligand. Second, the structure of the foreign gene can be tailored to encode the precise protein or nucleic acid product required to produce a specific effect. And third, the level of expression of the foreign gene can be predetermined through the selection of the gene's promoter.

4.A.2. Receptor Mediated Gene Transfer In the CNS: A Feasibility Study This research sought to determine the feasibility of using receptor-mediated gene transfer as a mechanism for introducing the expression of foreign genes in nerve cells. DNA carrier systems were constructed using neuronal ligands that are rapidly internalized by receptor-mediated endocytosis. These proteins, principally **wheat germ agglutinin** and **tetanus toxin C-fragment**, were complexed with high expression reporter genes and applied to nerve cells in vitro, and administered in vivo into rats. Uptake and expression of the reporter genes were analyzed by standard enzymatic and histochemical procedures.

4.A.3. Fundamental Outcomes and Conclusions This project has systematically tested the hypothesis that receptor-mediated endocytosis may serve as a means for targeting the delivery and expression of foreign genes in nerve cells. While we have demonstrated, for the first time, that cells in brain can internalize and express plasmid DNA, there is no evidence that this process can be made specific through the introduction of a receptor-mediated mechanism. The findings indicate that; (1) receptor-mediated uptake and expression does occur in the CNS,

and (2) lysosomal degradation is probably not the underlying basis for our inability to observe expression. From this it may be concluded that receptor-mediated uptake is not an efficient means for directing the expression of foreign genes in nerve cells.

4.A.4. Review of the Project

4.A.4.a. Objectives The overall objective of this research was to determine the extent to which neuron-specific ligands selectively target expression of foreign genes into neurons in vivo. The specific aims for the project were to:

1. Carry out the large scale production of reporter plasmid DNA and tetanus toxin C-fragment.

2. Develop optimal conditions for conjugating poly-L-lysine with the lectin, wheat germ agglutinin and tetanus toxin C-fragment. Wheat germ agglutinin, which is commercially available, served as a prototype ligand for subsequent work with C-fragment.

3. Optimize conditions for the synthesis of wheat germ agglutinin and tetanus toxin C-fragment targeted DNA carrier complexes.

4. Characterize ligand-mediated gene transfer in cells lines.

5. Characterize ligand-mediated gene uptake and expression in vivo.

4.A.4.b. Background in Brief

Our proposal to investigate neuron-specific gene transfer and expression was predicated on the findings in hepatocytes that demonstrate the practicality of this approach. Hepatocytes internalize asialoglycoprotein through receptor-mediated endocytosis.[4] Complexing asialoglycoprotein with a reporter gene (chloramphenicol acetyltransferase), placed under the control of a strong viral promoter (Simian virus 40), results in the targeted expression of the foreign gene specifically to hepatocytes bearing asialoglycoprotein receptors. Receptor negative cells do not take up the gene. Hepatocyte-specific gene transfer and expression is efficient and rapid, and occurs both in vitro and in vivo.[5-7] In rapidly dividing hepatocytes (induced by partial hepatectomy), the transferred DNA is integrated and expressed continuously for more than two weeks, the duration of the experimental period. Further, substitution of the viral promoter with that of a gene normally expressed in hepatocytes, albumin, results in the regulated expression of the fusion gene (Wu, personal communication).[8]

Targeting DNA transfer by a protein carrier has been demonstrated in several other systems. For example, DNA conjugated to a mitochondrial precursor protein is readily internalized across the mitochondrial membrane via a protein transport mechanism that closely resembles receptor-mediated endocytosis of the cell-surface membrane.[9] Together, these findings demonstrate the feasibility of using receptor ligands to target the transfer of DNA across cell membranes.

The cell membrane appears to be the principal barrier preventing the expression of exogenous genes. Once inside the intracellular compartment, however, foreign DNA is rapidly transported

to the nucleus where it is expressed by the same machinery that mediates the expression of the cellular genome. Although the precise mechanisms involved are still not clearly understood, it is well established that expression of a foreign gene is not dependent upon integration and can be selectively controlled by the gene's promoter.[10] Proteins encoded by foreign genes are correctly translated and generally undergo the posttranslational modifications that are necessary for biological activity. Thus, the cell membrane appears to be the primary barrier limiting the function of foreign genes in vivo.

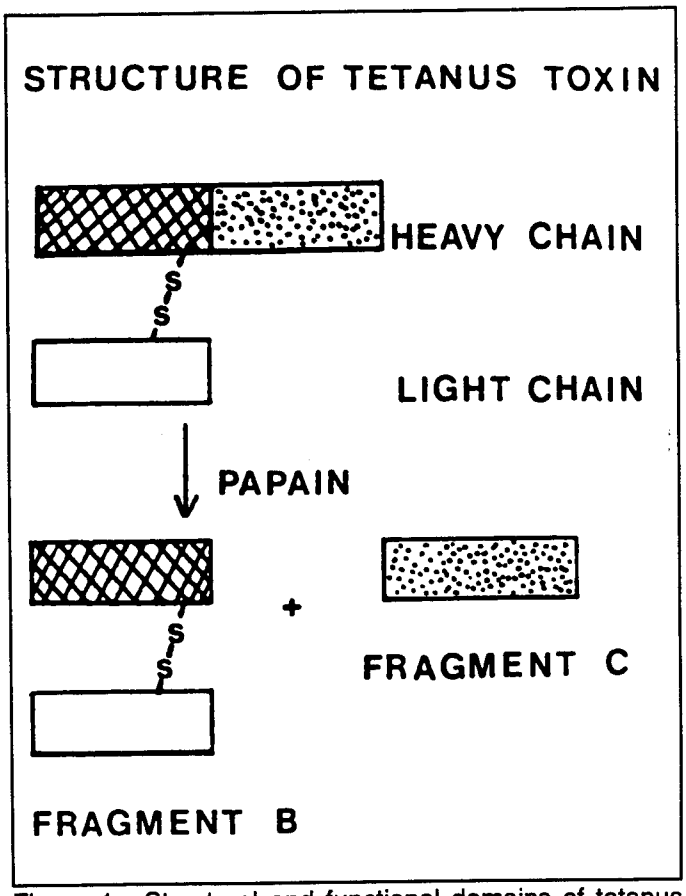

Figure 1. Structural and functional domains of tetanus toxin and their separation by limited proteolysis.

Neurons, similar to hepatocytes, rapidly internalize proteins through receptor-mediated endocytosis. Tetanus toxin and the lectin, wheat germ agglutinin, are well characterized examples. Both molecules are actively internalized after high affinity binding to complex gangliosides attached to membrane proteins.[11-16] The same basic mechanism, therefore, appears to mediate the uptake of both molecules into neurons. In the research conducted here, wheat germ agglutinin served as a prototype ligand for optimizing conditions for the use of tetanus toxin C-fragment. Though not as specific as C-fragment for neuronal uptake,[11] the lectin is readily available from commercial sources, whereas, bioactive C-fragment had to be prepared in-house specifically for the experiments discussed here.

Tetanus toxin is uniquely specific for uptake into neurons and enters the central nervous system from the circulation with the highest efficiency of any known protein.[17,18] Internalization occurs virtually immediately after the carboxy terminal (C-fragment) of the protein binds to high affinity receptors located on presynaptic terminals outside the blood-brain barrier; importantly, the toxic portion of the molecule resides in the amino terminal.[17,19] Once internalized the toxin is transported retrogradely to neuronal cell bodies, its presumed site of toxic action.[20,21] The toxin

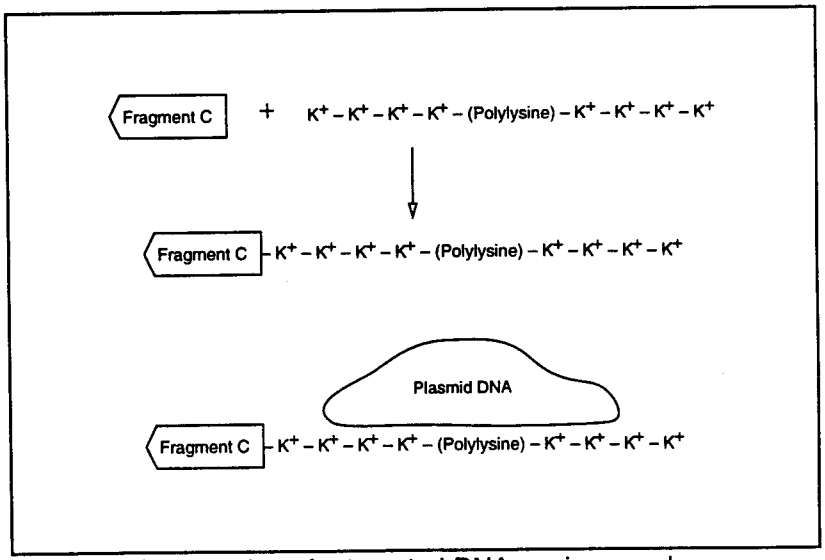

Figure 2. Construction of a targeted DNA carrier complex.

molecule is also passed from one neuron to the next by transsynaptic transport and this spread may contribute to the extreme toxicity of the holotoxin.[17,18,21-23] Retrograde and transsynaptic transport of the toxin mediate its transfer across the blood-brain barrier.

The C-fragment binding portion of tetanus toxin was separated from the holotoxin by proteolytic digestion as depicted in Figure 1 above. C-fragment alone is not toxic, yet, it is sufficient for internalization and transport, and therefore could be safely utilized as a carrier molecule for neuron specific gene transfer in vivo.[18,22,23] We hypothesized that genes complexed to the C-fragment of tetanus toxin will be taken up efficiently and specifically by neurons. And further, through expression, the foreign DNA will exert genetic control over specific neuronal functions.

A working model for the construction of targeted DNA carrier complexes is shown in Figure 2 above. The non-toxic portion of tetanus toxin (Fragment C) is covalently attached to the strongly cationic molecule, polylysine. The positive charge of the polylysine serves as a bridge for the non-covalent, electrostatic binding of negatively charged DNA. We hypothesized that the C-fragment would mediate receptor recognition and uptake for the entire complex into nerve cells.

4.B. SUMMARY OF THE MOST IMPORTANT RESULTS

4.B.1. Specific Aim #1: To Carry Out the Large Scale Production of Reporter Plasmid DNA and Tetanus Toxin C-Fragment.

Large scale production of plasmid DNA and tetanus toxin C-fragment were accomplished using what are now standard procedures. Our studies primarily involved the PUC-based RSV-β-GAL construct replicated in *E. coli* to produce in milligram quantities of reporter gene plasmid. C-fragment was prepared by a limited tryptic digestion. As depicted in Figure 1 above, the cell binding (C-fragment) and cytotoxic (B-fragment) portions of tetanus toxin are highly resistant to proteolytic attack, whereas, the flexible linker peptide that joins the two is not.

Figure 3. Intracellular localization of internalized C-fragment within brain neurons.

As such, the two functional elements are readily separated by limited digestion with either papain or trypsin. Our work primarily involved the use of trypsin which is resistant to self-degradation and available in very high purity. The non-toxic C-fragment was then purified from the digest reaction mixture by either gel filtration or fast protein liquid chromatography (FPLC). Preparations of plasmid and C-fragment were demonstrated to be active, respectively, by the

formation of blue X-GAL reaction product in bacterial (not shown) and by uptake into the central nervous system Figures 3-5).

The electron micrograph in Figure 3 shows the high concentration of C-fragment into presynaptic vesicles and synaptic clefts of motor neurons. The basis for the uptake and transport of C-fragment is its binding to complex gangliosides which are present in high concentrations on neurons and particularly in synapses. This unique property of C-fragment is also the basis for its passage from neuron to neuron. Figure 4 shows a section of midbrain take from a mouse treated with C-fragment coupled to the marker enzyme horse radish peroxidase (HRP). C-fragment-horse radish peroxidase complex was administered systemically in a dose of 0.2 mg and samples collected 4 days later. Equivalent uptake is not seen with free HRP administered in doses as great as 40 mg. Even though C-fragment enters the CNS at discrete entry points, it spreads transcellularly from the cells which initially transport it from the periphery. Figure 5 shows the extent of labeling of spinal cord grey matter 4 days after an intramuscular injection of C-fragment on the right side. This pattern of uptake and passage is very different from that of most other proteins which are rapidly degraded by lysosomes within the cells that incorporate them. The synaptic, non-lysosomal, localization of the C-fragment together with its transsynaptic passage are thought to mediate the long life (over 7 days) of protein in the CNS.

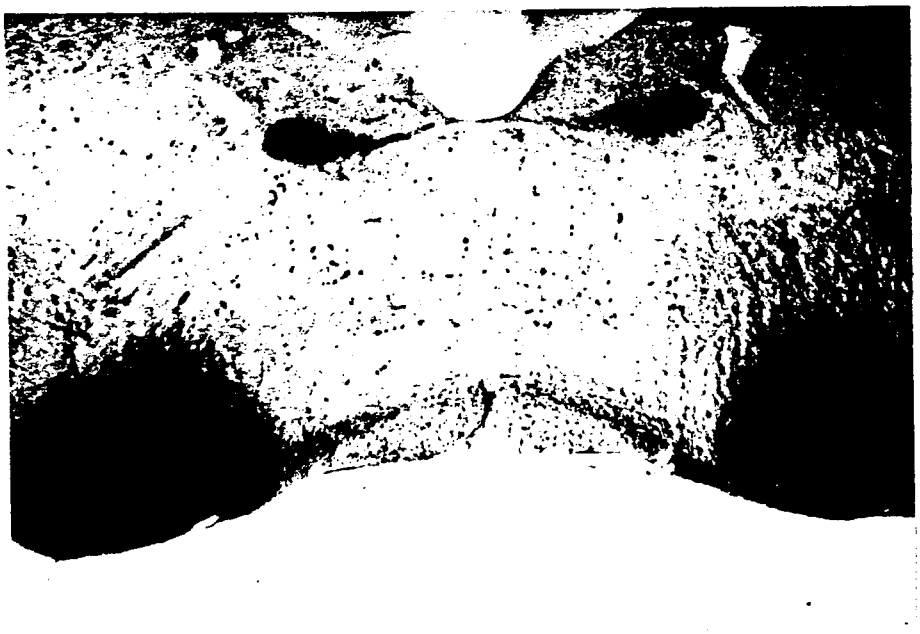

Figure 4. Uptake of C-fragment-HRP into the midbrain of the CNS.

Figure 5. Uptake and transsynaptic transport of HRP labeled C-fragment into the spinal cord.

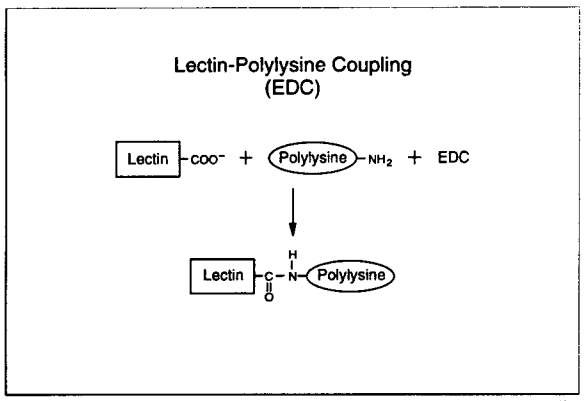

Figure 6. 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide (EDC) coupling of -NH_2 and -COOH groups.

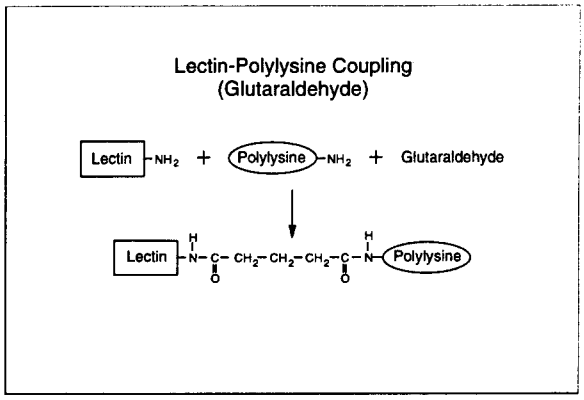

Figure 7. Glutaraldehyde coupling of NH_2 pairs.

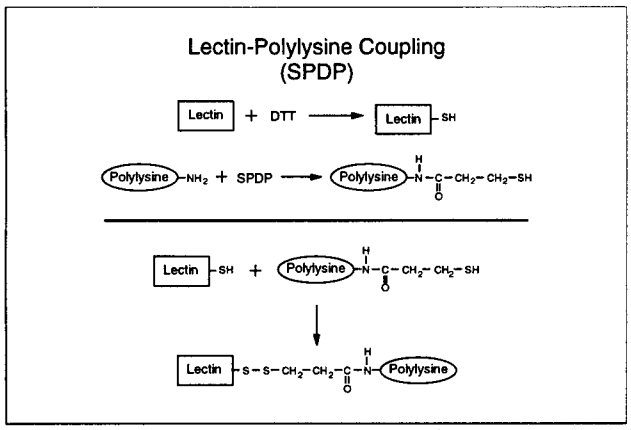

Figure 8. Formation of a mixed disulfide by reduction with dithiothreitol (DTT) and coupling with N-hydroxysuccinimidyl-2,3-dibromoproprionate (SPDP).

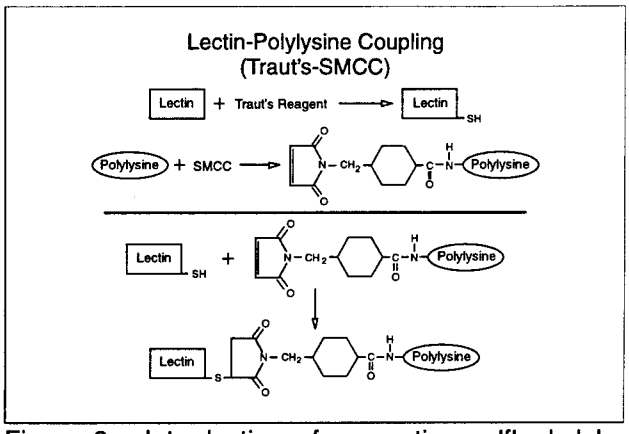

Figure 9. Introduction of a reactive sulfhydryl by Traut's reagent and a maleimide by succinimidyl 4[N-maleimidomethyl]cyclohexe-1-carboxylate (SMCC).

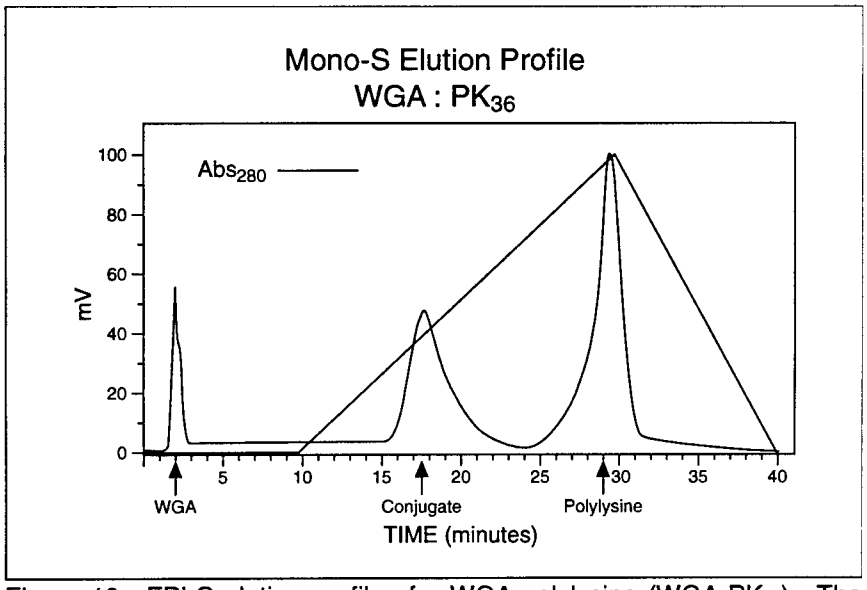

Figure 10. FPLC elution profile of a WGA-polylysine (WGA-PK_{36}). The peaks represent absorbance at 280 nm, the linear line indicates the Na^+ elution gradient.

4.B.2. Specific Aim #2: To Develop Optimal Conditions for Conjugating Poly-L-Lysine with Wheat Germ Agglutinin and Tetanus Toxin C-Fragment. Covalent coupling of wheat germ agglutinin and C-fragment with poly-L-lysine proved to be a greater challenge than anticipated. Four different approaches were evaluated in the course of developing what we consider to be an effective process. The reactions involved are shown in Figures 6-9. Although each of the four approaches produced conjugated product. The reaction mediated by EDC proved to be the most effective. The reaction product was purified by FPLC on a strong cation exchange column (MONO-S) as shown in Figure 10. Based upon the relative absorbance at 280 nm we estimated that the average coupling efficiency for the reaction was approximately 30%. The product was stable at 4°C for up to 4 days as judged by repeated FPLC analysis. Freezing resulted in condensation and complete loss of functional conjugate.

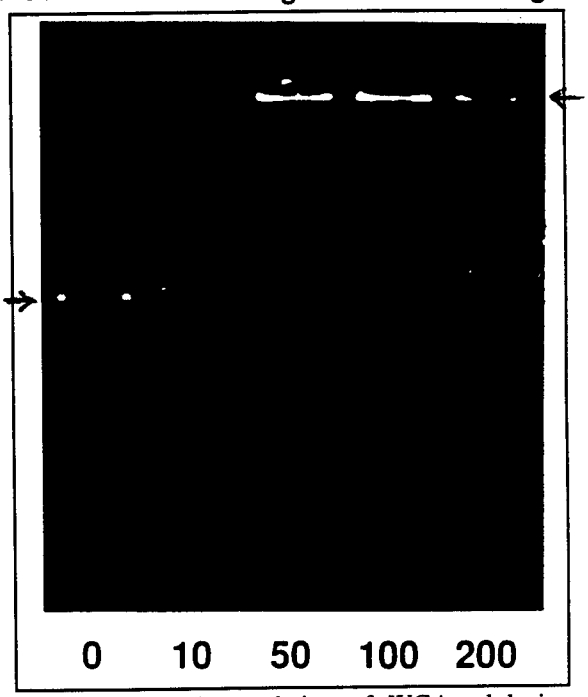

Figure 11. Gel retardation of WGA-polylysine conjugates (#'s indicate ug/ml lectin-polylysine)

4.B.3. Specific Aim #3: Optimize Conditions for the Synthesis of Wheat Germ Agglutinin and Tetanus Toxin C-fragment Targeted DNA Carrier Complexes.

The ability of polylysine and wheat germ agglutinin or C-fragment complexes to carry DNA was determine in a gel shift assay. In this assay plasmid DNA or DNA molecular weight standards were incubated in the presence of the polylysine conjugates and then subjected to agarose electrophoresis. Ethidium bromide staining was used to visualize the migration of the DNA and determine the relative amount that was retarded by interaction with the polylysine-based conjugates. The gel shown in Figure 11 demonstrates how the proportion of DNA to conjugate can be titered to achieve optimal proportions for the most efficient interaction. In this experiment 25 µg of plasmid DNA was incubated in the presence of increasing concentrations of lectin-polylysine conjugate. Complete retardation is evident at a concentration of 50 µg/ml carrier. These data are representative of all conjugates prepared including those tested for interaction with radiolabeled DNA (^{32}P nick translated; versus

Table 1. Model Systems for Studying Receptor-Mediated Events

Cells for Gene Transfer *in vitro*	
PC12	
Neural Origin	Rat pheochromocytoma, Adrenergic
Receptors	Tetanus toxin, NGF, Lectin, Transferrin
NS20Y	
Neural Origin	Mouse neuroblastoma, Cholinergic
Receptors	D1, Opiate, Lectin, Transferrin
3T3	
Neural Origin	Mouse
Receptors	Lectin, Transferrin

those visualized by ethidium bromide staining.

4.B.4. Specific Aim #4: To Characterize Ligand-Mediated Gene Transfer in Cells Lines.

Uptake of targeted carrier complexes were first evaluated in cell culture. This work was carried out in the cell lines listed in Table 1. As indicated, each line is well characterized for receptor-mediated endocytosis. Figure 12 shows the time course for wheat germ agglutinin uptake into PC12 cells. In this experiment the lectin was radioiodinated with ^{125}I and incubated with the cells for the times indicated. Because different cell lines exhibit different uptake characteristics, this type of analysis was done for each cell line and ligand. Figure 13. shows the ability of lectin to mediate the uptake of ^{32}P-nick translation labeled plasmid DNA into cells. The uptake was shown to be time and temperature dependent and specific for receptor-mediated endocytosis as judged by competition studies with unlabeled ligand.

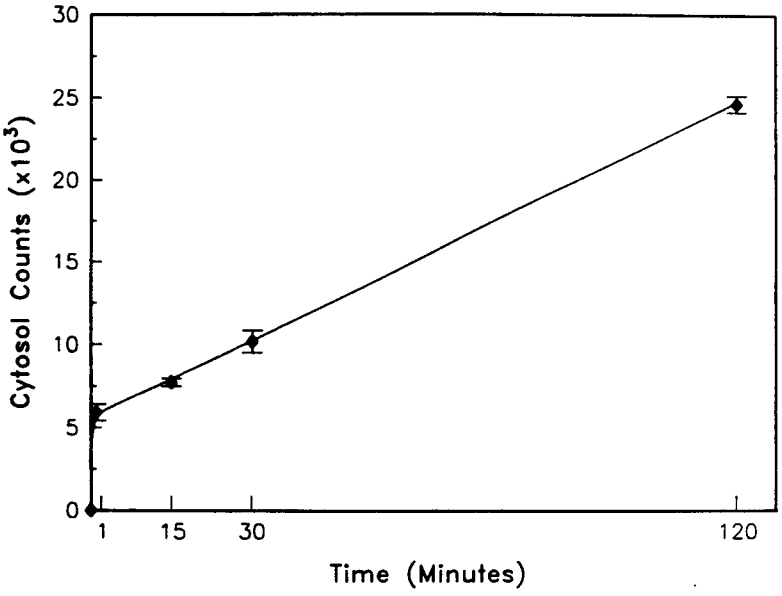

Figure 12. Representative time course for the uptake of ^{125}I labeled lectin by PC12 cells in culture.

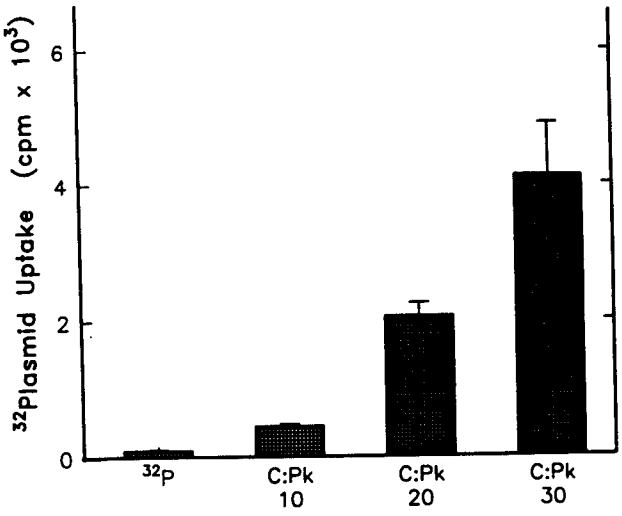

Figure 13. Lectin mediated Uptake of ^{32}P labeled plasmid DNA into 3T3 cells. Numbers = ug/ml carrier

4.B.5. Characterize Ligand-Mediated Gene Uptake and Expression *In Vivo*

The uptake and expression of foreign DNA in the CNS of rats is mediated by liposomes. Liposome encapsulated plasmid DNA was administered directly into the cerebral venticals of rats and samples collected 1-7 days later. As Figures 14-16 show at 4 days, expression of β-GAL is concentrated in the vasculature of the circumventricular organs and occurs diffusely throughout the parenchyma. We believe that these findings were first demonstration that plasmid DNA can be expressed in brain. Though novel and interesting, these findings were viewed as controls for studies on ligand-mediated receptor uptake and expression. Although the findings demonstrate convincingly that foreign DNA can be expressed in brain under experimental conditions, the expression was neither targeted nor regulated.

GAL reporter plasmid DNA was mixed (1:10 and other ratios) with polylysine carrier complex. C-fragment, wheat germ agglutinin, concanavalin A, were all evaluated as targeting ligands. The carrier complexes were administered directly into the lateral ventricle and samples collected 2-4 days later, a period in which the expression of plasmid DNA was readily detected in the experiments using liposomes discussed under 4.B.5 above.

The results of these experiments were completely negative. There was no evidence for receptor-mediated uptake and expression of RSV-β-GAL under any of the conditions.

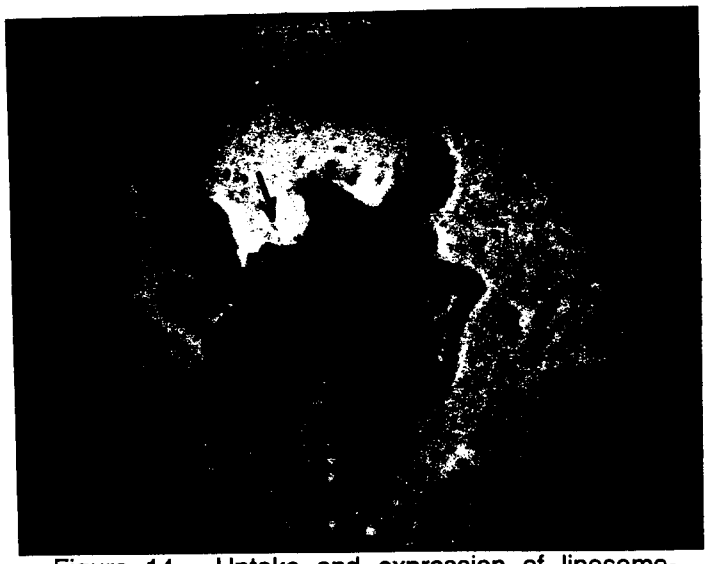

Figure 14. Uptake and expression of liposome-carried plasmid DNA in choroid plexus.

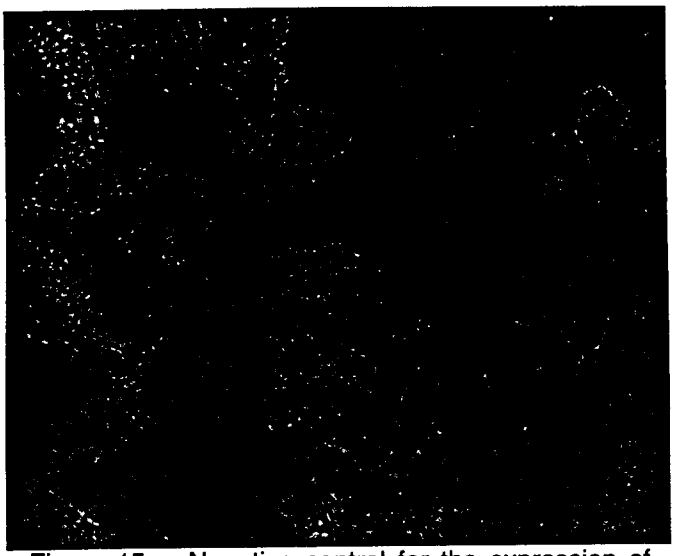

Figure 15 . Negative control for the expression of RSV-β-Gal in choroid plexus.

Figure 16. Uptake and expression of liposome carried DNA in the median eminence.

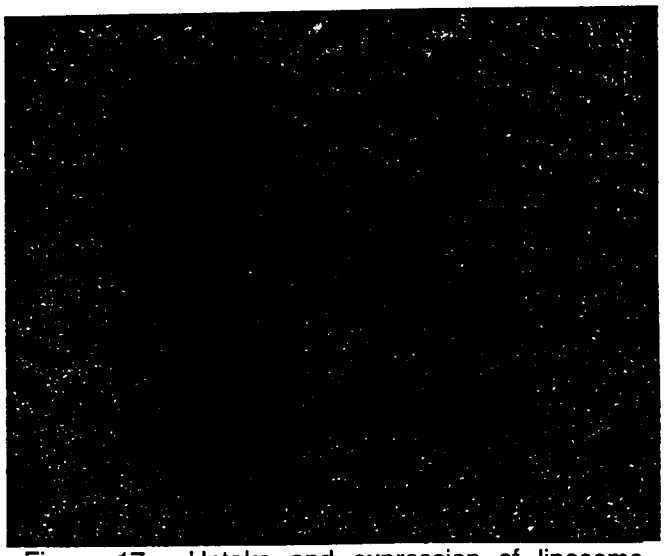

Figure 17. Uptake and expression of liposome carried DNA in cells of the cerebral cortex.

4.B.5.b. Targeted Expression of Chloramphenicol Acetyltransferase These negative findings prompted us to test the more sensitive, radioenzymatic and luminescence reporter systems. The genes encoding the enzyme chloramphenicol acetyltransferase (CAT) or firefly luciferase were substituted for RSV β-GAL and a similar set of experiments conducted. The *in vitro* CAT and luciferase assays were optimized for detecting expression. It is generally held that these detection systems are between 10- and 100-fold more sensitive than the detection of β-GAL. A series of tests were carried out in cell culture using the NS20Y cell line which expresses CAT and luciferase very well. A representative experiment with CAT is shown in Figure 17.

Again, the results of these experiments were completely negative. There was no evidence for receptor mediated uptake and expression of CAT any of the conditions.

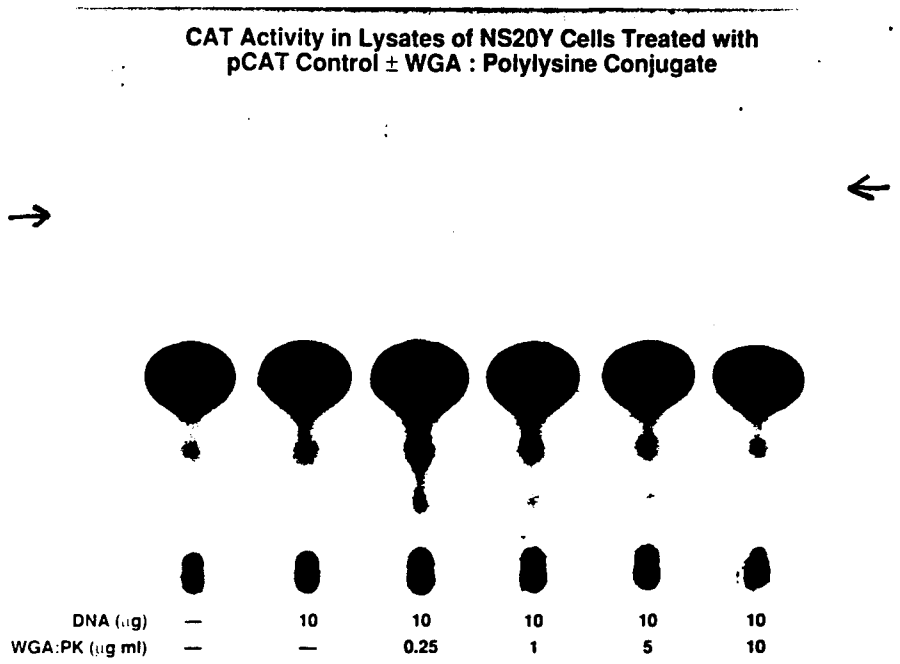

Figure 17. Inability of WGA to mediate the uptake and expression of CAT in NS20Y cells.

4.B.5.c. Adenovirus and a Mechanism to Prevent Lysosomal Degradation Targeted DNA Carrier Complexes. We next sought to determine if the failure to detect foreign gene expression resulted from intracellular degradation of the complexes by lysosomes. Characteristically, the process of receptor-mediated uptake results in the formation of an endosome which is then targeted to the lysosomal compartment where it is degraded and parts recycled. Recently, it was reported by Curiel and coworkers (*PNAS 88: 8850, 1991*) that infection with adenovirus disrupts lysosomes and greatly enhances the expression of reporter genes in human HELA cells. These findings were made with a replication-incompetent adenovirus (dl312) that is unable to mount a lytic infection yet still acutely disrupts lysosomal function. This original report has triggered several investigations which have now demonstrated the utility of adenovirus to enhance targeted gene delivery without interfering with specificity.[24,25] A working model for this strategy is presented in Figure 18 above.

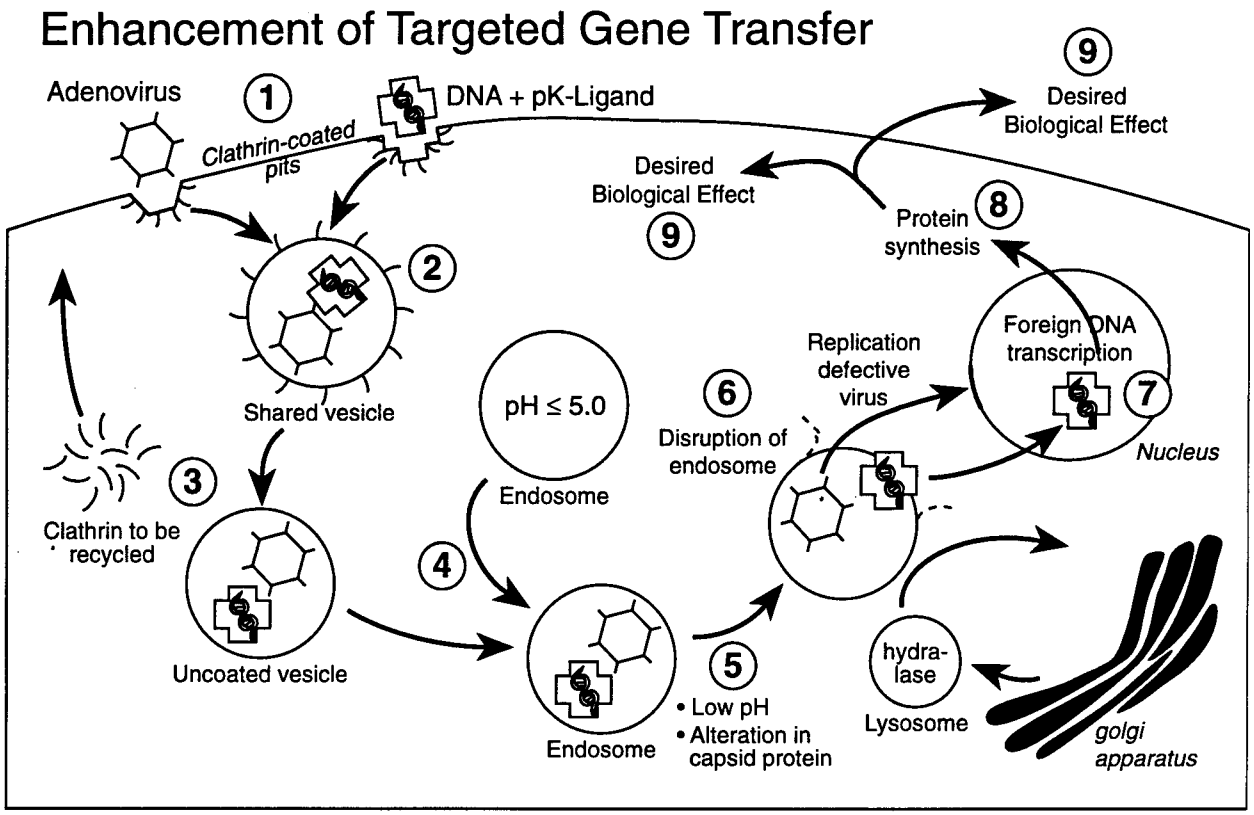

Figure 18. Infection with a replication defective adenovirus as a means to prevent lysosomal degradation of endocytic vesicles.

Our next step sought to determine if replication-incompetent adenovirus could enhance foreign gene expression in our rodent cell models. Towards this goal we obtained the dl312 strain of adenovirus from Dr. Curiel for evaluation. The virus was administered as intracerebroventricularly together with RSV-β-GAL/C-fragment carrier complex and samples collected 4 days later.

Again there was no evidence for receptor-mediated uptake and expression. From this it may be concluded the lysosomal degradation is probably not the principal cause for the absence of foreign gene expression in these experiments.

4.B.5.d. Synthesis of C-Fragment/Polylysine/HRP Complex.

Our final effort for the project has focused on the production of carrier complexes that contain the reporter enzyme HRP, as well as plasmid DNA. Our intention was to use HRP as another approach for assessing internalization, one that did not depend upon the expression of foreign DNA. As such, we could study the processes of uptake and expression independently. Progress in this effort has resulted in the production of the complexes as judged by gel shift in SDS PAGE. In this work the lectin horse radish peroxidase was first mixed with sodium periodate and then incubated in the presence of C-fragment and polylysine in a 200 mM carbonate buffer, pH 9. Lane 1 = C-fragment, lane 2 = horse radish peroxidase; lane 3 = polylysine; lane 4 = conjugate. The conjugate (lane 4) was largely retained within the sample

well, unable to enter the gel due to its size. The limits of time and technical assistance have precluded further characterization of the product for DNA binding or biologic activity in cell culture and *in vivo*.

4.B.5.e. Summary and Conclusions We have systematically tested the hypothesis that receptor-mediated endocytosis may serve as a means for targeting the delivery and expression of foreign genes in nerve cells. While we have demonstrated, for the first time, that cells in brain can internalize and express plasmid DNA, there is no evidence that this process can be made specific through the introduction of a receptor-mediated mechanism. The findings indicate that; (1) receptor-mediated uptake and expression does occur in the CNS, and (2) lysosomal degradation is probably not the underlying basis for our inability to observe expression. From this it may be concluded that receptor-mediated uptake is not an efficient means for directing the expression of foreign genes in nerve cells.

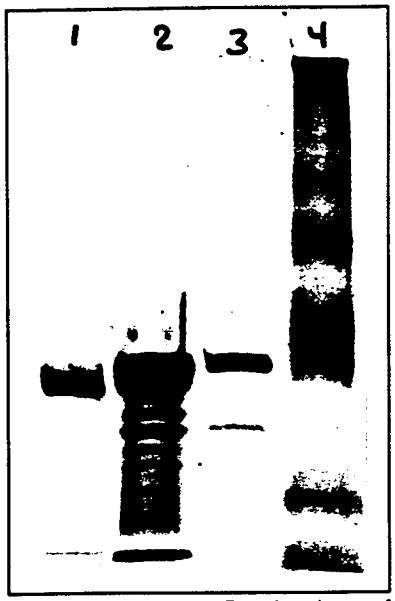

Figure 19. Synthesis of polylysine/C-fragment/horse radish peroxidase conjugate.

4.C. List of Publications and Technical Reports.

Gregory P. Mueller, Ph.D. and Paul S Fishman, M.D.: Receptor-mediated uptake and expression of foreign genes in nerve cells. In: Signal Transduction and Information Processing in Biological Systems", A workshop-symposium sponsored by the US Army Research Office, 26-29 April 1992, High Hampton Inn, Cashiers, NC.

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5. Report of Inventions None

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